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Cell cycle regulatory proteins in glomerular disease

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Cell cycle regulatory proteins in glomerular disease. Evidence is accumulating that directly responsible for the rate of progression of glomerular disease are specific positive (cyclins and cyclin-dependent kinases) and negative (cyclin-kinase inhibitors) cell cycle regulatory proteins. The challenge for nephrologists is to determine which ones are expressed in renal disease and their precise role in glomerular cell proliferation, hypertrophy and differentiation. Ultimately the goal is to find ever more appropriate therapeutic strategies to arrest or prevent progressive renal disease.

There are many forms of glomerular growth that characterize different types of glomerular diseases, and these include proliferation, hypertrophy, and differentiation. As is discussed later in this article, each type of glomerular growth underlies the development of progressive glomerulosclerosis, and thus, as nephrologists, it is of fundamental importance that we understand and can intervene with these processes to reduce progressive disease. There are multiple potential mediators of glomerular growth, such as growth factors and their receptors, signaling pathways, and transcription factors. However, there is increasing evidence to show that regulation of the cell cycle by cell cycle regulatory proteins, which are predominantly located in the nucleus, ultimately determines the growth response to injury.

Over the past decade, particularly the past few years, three paradigms have emerged in the study of the cell cycle. First, each phase of the cell cycle serves a specific function in preparing the cell to proliferate [1]. Thus, in G_0 phase of the cell cycle, cells such as normal glomerular cells are quiescent [2]. Proliferation requires that cells engage the cell cycle at early G_1 and progress through and complete each phase of the cell cycle. In preparing the cell to proliferate, protein synthesis increases in G_1. DNA synthesis occurs in the S phase, and cells undergo mitosis and cytokinesis (cell division) in the M phase (Fig. 1).

Second, the original view that the cell cycle only governs cell proliferation is no longer valid because under certain circumstances, arrest in or exit from the cell cycle results in different types of growth (Fig. 1). Thus, arrest in G_1 is associated with hypertrophy [3]. Exit from the cell cycle in late G_1 leads to apoptosis [4], and the programmed exit from the cell cycle at G_2/M is associated with cell differentiation [5].

Third, each phase of the cell cycle is controlled by specific positive [cyclins and cyclin-dependent kinases (CDKs)] [6, 7] and negative (cyclin-kinase inhibitors) cell cycle regulatory proteins (Fig. 2) [8]. Detailed reviews of cell cycle proteins has been presented by others at this symposium and in other reviews. In brief, progression through or arrest during the cell cycle is controlled by cell cycle regulatory proteins. Proliferation (completion of the cell cycle) requires that cyclins bind to and activate specific CDKs in each phase of the cell cycle (Fig. 2). In contrast, the inhibition of cyclin-CDK complexes by cyclin-kinase inhibitors inhibits proliferation by causing cell cycle arrest.

The study of cell cycle regulatory proteins in glomerular disease poses a number of interesting challenges for nephrologists. These include determining: (*a*) which specific cell cycle proteins are expressed in glomerular disease; (*b*) the role of cell cycle proteins in glomerular cell proliferation, hypertrophy, and differentiation; (*c*) whether or not these findings are specific to glomerular cells and how they differ from nonrenal cells; (*d*) adapting what has been learned in renal and nonrenal cell culture studies to experimental and human glomerular disease; and (*e*) satisfying the ultimate goal, which is to identify specific cell cycle proteins as therapeutic targets in glomerular disease.

MESANGIAL CELL PROLIFERATION

There is now abundant evidence to show that cell proliferation is central to several types of glomerular diseases [9], thus making the potential to alter the proliferative response of great interest to both clinical and

Key words: glomerular growth, cell proliferation, hypertrophy, progressive glomerulosclerosis.

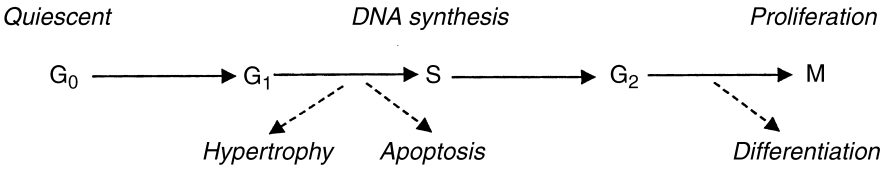

Fig. 1. Phases of the cell cycle. The cell cycle is divided into the G_0 (quiescent), S (DNA synthesis), and M (mitosis) phases, which are separated by the resting phases called G_1 and G_2. Proliferation requires that cells progress through the cell cycle. Arrest in G_1 can result in inhibition of proliferation or, under different circumstances, hypertrophy. Exit from the cell cycle in late G_1 results in apoptosis, whereas cells exit in G_2 to undergo differentiation.

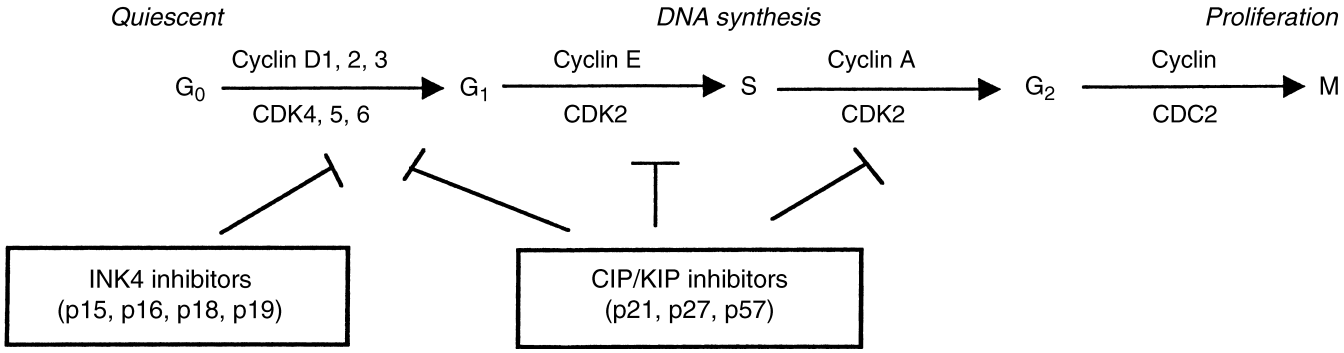

Fig. 2. Cell cycle regulatory proteins. Progression through each phase of the cell cycle requires that the positive cell cycle proteins cyclins bind to and activate a specific cyclin-dependent kinase (CDK). In contrast, proliferation is inhibited by negative cell cycle proteins called cyclin kinase inhibitors. The INK4 inhibitors are restricted to G_1 cyclin-CDK complexes, whereas the CIP/KIP inhibitors inhibit G1 and S phase cyclin-CDK complexes.

experimental nephrologists. A characteristic response to immune [IgA nephropathy, mesangial proliferative glomerulonephritis (MPGN), lupus nephritis] and nonimmune (remnant kidney)-mediated glomerular injury is mesangial cell proliferation [10]. The importance of mesangial cell proliferation has been shown in experimental and human glomerular disease, where mesangial cell proliferation precedes and often predicts increased mesangial matrix production [11]. Moreover, maneuvers that reduce mesangial cell proliferation such as low-protein diet [12], complement depletion [13, 14], blocking the cytokines platelet-derived growth factor (PDGF) [15], basic fibroblast growth factor (bFGF), and heparin [16], inhibiting the PDGF receptor with trapidil [17], and inhibiting specific intracellular signaling pathways with phosphodiesterase inhibitors [18] have also been associated with the reduction in mesangial matrix production. However, we have focused on the role of cell cycle proteins in mesangial cell proliferation because of the compelling view that cell proliferation is ultimately controlled in the nucleus by these proteins.

Cyclins and cyclin-dependent kinases in mesangial-cell proliferation

We began our studies by examining the expression of specific cell cycle proteins in experimental glomerular disease. Experimental mesangial proliferative glomerulonephritis (Thy1 model) is induced by an antibody directed against the Thy1 antigen normally present on rat mesangial cells, which results in a complement-dependent injury to the mesangial cell (mesangiolysis) [19, 20]. This is followed by a phase of marked mesangial cell proliferation, which precedes and is closely associated with the development of matrix accumulation and a decline in renal function [13]. The Thy1 model of mesangial proliferative glomerulonephritis is therefore a very useful model to further our understanding of the mechanisms underlying the mesangial cell's proliferative response to injury.

The onset of mesangial cell proliferation in Thy1 glomerulonephritis is associated with a significant increase in immunostaining for cyclin A [21]. The catalytic partner of cyclin A is CDK2, which is required and essential for DNA synthesis [22, 23]. In contrast to quiescent nonrenal cells where CDK2 is normally constitutively expressed throughout the cell cycle, CDK2 immunostaining was absent in the normal quiescent rat glomerulus [21]. However, mesangial cell proliferation in experimental glomerulonephritis is associated with a marked increase in glomerular immunostaining for CDK2, and double immunostaining with cell-specific markers showed that CDK2 localizes to mesangial cells [21]. To show that CDK2 was functionally active, glomeruli were isolated from Thy1 animals, and protein was extracted to measure

CDK2 activity by the histone H1 kinase assay. Our data showed that CDK2 activity was markedly increased during mesangial cell proliferation *in vivo* [21]. In other experimental models associated with mesangial cell proliferation such as the remnant kidney model [24], CDK2 protein is also increased. More recently, we have shown that CDK2 immunostaining is also increased during the proliferative phase following endothelial cell injury in experimental thrombotic microangiopathy (unpublished data). Moreover, CDK2 protein and activity are also increased during growth factor-induced mesangial cell proliferation *in vitro* [25, 26].

Showing that both the expression and activity for CDK2 were increased in experimental glomerulonephritis suggested that this cell cycle protein may be a potential target for therapeutic intervention in diseases characterized by mesangial cell proliferation. To this end, we studied the purine analogue roscovitine [27–29] and showed that roscovitine causes a dose-dependent reduction in CDK2 activity without altering CDK2 proteins levels in cultured mesangial cells [26]. Furthermore, inhibiting CDK2 activity by roscovitine completely abrogated growth factor-induced mesangial cell proliferation *in vitro* [26]. To determine the role of CDK2 in mesangial cell proliferation *in vivo*, roscovitine was given as daily intraperitoneal injections to Thy1 animals, which resulted in a significant reduction in the glomerular activity for CDK2 compared with control animals. Importantly, roscovitine did not interfere with the binding of Thy1 antibody, complement activation, or the influx of infiltrating cells such as macrophages and platelets during the early phase of inflammation. Our results showed that the early decrease in CDK2 activity was associated with a marked reduction in the peak (day 5) of mesangial cell proliferation and glomerular cellularity in Thy1 nephritis [26].

As discussed earlier, previous studies have established a relationship between mesangial cell proliferation and matrix production [9]. Our results showed that the decrease in mesangial cell proliferation induced by roscovitine was also associated with a significant decrease in the extracellular matrix proteins collagen type IV, laminin, and fibronectin [26]. Moreover, renal function was significantly improved compared with control animals. What happens if CDK2 activity is inhibited once mesangial cell proliferation has already been established rather than prior to the onset of proliferation? To answer this, Thy1 rats were given roscovitine starting at day 3 when proliferating cell nuclear antigen and BrdU, markers of DNA synthesis, were already increased. When given under these conditions, roscovitine also significantly reduced mesangial cell proliferation at later time points in Thy1 [26].

These studies demonstrated that CDK2 activity could be inhibited *in vivo* without altering the level of the protein. Furthermore, decreasing CDK2 activity profoundly inhibited glomerular cell proliferation, which was associated with an improvement in renal function. With newer and more potent pharmacological agents being developed that target specific cell cycle proteins, CDK2 should be considered as a potential therapeutic target for inflammatory diseases characterized by proliferation.

Cyclin kinase inhibitors in glomerular cell proliferation

Cyclin kinase inhibitors (CKIs) inhibit cell proliferation by binding to and inactivating specific cyclin-CDK complexes [8, 30, 31]. There are two families of CKIs that are based on sequence homology and the phase of the cell cycle in which they inhibit cyclin-CDK complexes (Fig. 2). We have focused on the Cip/Kip family of CKIs, which includes p21, p27, and p57, because these CKIs inhibit early G_1 (D-type cyclin-CDK4, 6), late G_1 (cyclin E-CDK2), and S-phase (cyclin A-CDK2) cyclin-CDK complexes. What has become increasingly evident is that the distribution and function of these CKIs are not predictable or expected and are cell-type specific. There is a differential expression of CKIs in the normal quiescent glomerulus. Immunostaining for p21 is absent in normal glomeruli, whereas p27 is constitutively expressed in all three resident glomerular cell types [21]. In contrast, immunostaining for $p57^{Kip2}$ (p57) localizes exclusively to glomerular epithelial cells (GECs; podocytes).

Similar to what has been shown in the mesangial cell *in vitro* [32], mesangial cell proliferation *in vivo* in the Thy1 model is associated with a marked decrease in levels for p27 [32]. Moreover, by day 5, which is the peak of mesangial cell proliferation in this model, p27 levels are barely detectable. However, during the recovery phase of mesangial cell injury, where proliferation ceases, p27 levels returned to baseline. Of note is that the decrease in levels of p27 during mesangial cell proliferation was not a predictable finding because p27 levels remain unchanged during proliferation in other cell types such as vascular smooth muscle cells (M. Reidy, personal communication).

In contrast to p27, there is *de novo* synthesis of the CKI $p21^{Cip1,WAF1}$ (p21) during Thy1 glomerulonephritis, and double immunostaining showed that p21 localized to mesangial cells [32]. Furthermore, the increase in p21 expression coincided with the resolution phase of mesangial cell proliferation in this model. Further studies are being undertaken to determine whether this CKI is required for the resolution of mesangial cell proliferation in disease.

We have used a genetic approach to establish the functional role of p27 in glomerular cell proliferation. Experimental glomerulonephritis was induced in p27 wild-type (+/+) and p27 knockout (−/−) mice [33], and the study

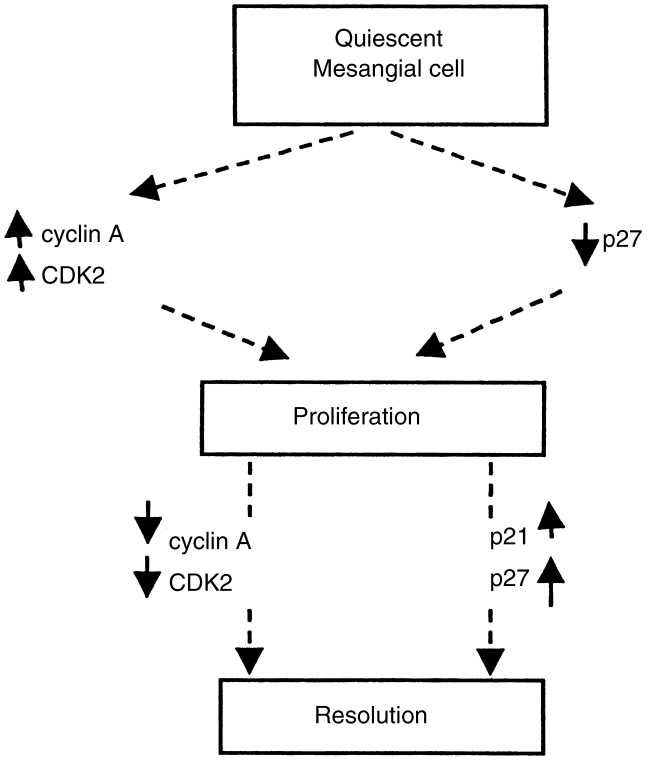

Fig. 3. Mesangial cell proliferation. The onset of mesangial cell proliferation requires increased expression and activity of cyclin A-CDK2. The levels of the cyclin kinase inhibitor p27 determine the proliferative threshold so that proliferation is augmented when p27 levels decrease. The resolution of proliferation requires a decrease in CDK2 activity, which may be due to a decrease in cyclin A and CDK2 expression and/or an increase in levels of the p21 and p27.

showed three findings. First, under normal physiological conditions, the loss of p27 by itself was not associated with ongoing DNA synthesis in adolescent p27−/− mouse kidneys. Second, during inflammatory glomerulonephritis, the onset of glomerular cell DNA synthesis and proliferation occurred at earlier time points in p27−/− mice compared with controls. Third, in glomerulonephritis, the magnitude of glomerular DNA synthesis and proliferation was markedly increased at all time points studied in p27−/− mice compared with controls. To ensure that the increase in glomerular cell proliferation in p27−/− mice was not due to a difference in immune-response, p27+/+ and p27−/− mice underwent total body irradiation, and the bone marrow was reconstituted with marrow from normal p27+/+ mice. Experimental glomerulonephritis was then induced in both groups of marrow-transplanted mice. The results confirmed that glomerular cell proliferation was increased in nephritic p27−/− mice compared with controls, and also demonstrated that the levels of p27 in the glomerulus rather than the immune response determine the proliferative response to injury.

A summary of the role for cell cycle proteins in mesangial cell proliferation is shown in Figure 3. Mesangial cell proliferation requires an increase in expression and activity of the positive cell cycle proteins cyclin A-CDK2. However, the onset and magnitude of mesangial cell proliferation are determined by the CKI p27, which supports the idea that p27 sets the threshold at which proliferation occurs.

VISCERAL GLOMERULAR EPITHELIAL CELL PROLIFERATION AND DIFFERENTIATION

Glomerular epithelial cells exit the cell cycle during glomerulogenesis

The mature visceral GEC or podocyte is a unique glomerular cell because its growth response to injury differs from the mesangial and endothelial cells by virtue of its apparent inability to proliferate [34]. However, in contrast to mature GECs, immature GECs proliferate during specific stages of glomerulogenesis [35]. Thus, during the S-shaped stage, undifferentiated glomerular cells that transform into GECs are characterized by marked proliferation. However, during the comma stage of glomerular development, GECs exit the cell cycle and become terminally differentiated (Fig. 1), a phenotype that is quiescent.

We speculated that the GEC exit from the cell cycle during glomerulogenesis was due to specific CKIs. To test this hypothesis, we studied human glomerulogenesis in which both proliferating and nonproliferating cell populations can easily be identified in the same kidney. Immunostaining for the CKI p27 was absent in proliferating immature cells that become GECs during the S-shaped stage of glomerular development. In contrast, the cessation of GEC proliferation that accompanies GEC exit from the cell cycle coincided with the *de novo* expression of p27 [36]. These results suggest that p27 may be required for normal GEC differentiation.

What about the expression of other CKIs during GEC development? p57 is also expressed *de novo* in GECs during mouse and rat glomerulogenesis. A role for p57 in GEC biology has been shown in genetic studies. Animals that lack p57 (p57 knockout mice) are born with fused foot processes and have many features that resemble Beckwith–Wederman syndrome [37]. These studies suggest that p57 may be required for normal GEC development. In contrast, mice lacking the CKI p21 (p27−/− mice) have normal kidneys, suggesting that p21 is not essential for normal GEC differentiation. Taken together, specific CKIs may be essential for the development of the quiescent differentiated GEC phenotype.

Inability of mature differentiated glomerular epithelial cell proliferation

Although there is some debate regarding the proliferative capacity of mature GECs, most studies have shown that in striking contrast to mesangial and endothelial

cells, GEC proliferation is not marked. However, under certain circumstances, GECs do re-enter the cell cycle and undergo DNA synthesis, albeit low grade compared with mesangial cells, and proliferate [38]. However, the current paradigm is that the relative inability of the GECs to proliferate may underlie the development of glomerular sclerosis in many forms of injury to the GECs [34, 39, 40].

Many differentiated cells can re-engage the cell cycle under certain cues. Why then do mature GECs not proliferate? One thesis is that terminally differentiated GECs lack certain positive cell cycle (cyclins and CDKs) proteins required to progress through the cell cycle. Another possibility is that the levels of negative cell cycle proteins (CKIs) increase. To address these possibilities, we studied the passive Heymann nephritis (PHN) model of membranous nephropathy, which is induced by the administration of an antibody to the anti-Fx1A antigen on the GECs, resulting in a complement-dependent injury to GECs [41, 42]. This is followed by the formation of subepithelial immune deposits, foot process fusion and effacement, and proteinuria. In contrast to the increase in mesangial cell number following complement-induced injury to the mesangial cell, the GEC number is normal in experimental membranous nephropathy [43]. Studies have shown that this is due to an apparent inability of GECs to proliferate. The significance of the apparent inability of GECs to proliferate has been proposed by Kriz and Rennke [34, 39, 44] to involve the inability of GECs to replace injured and lost GECs leading to a denuded glomerular basement membrane, which underlies the development of progressive glomerulosclerosis.

To determine if the inability of GECs to proliferate is due to the lack of cell cycle proteins required for DNA synthesis, the expression for cyclin A and CDK2 was measured. Complement-dependent GEC injury in PHN was followed by a small but significant increase in immunostaining for cyclin A and CDK2, and this localized to GECs [45]. These results suggested that GECs have the "nuclear machinery" required for DNA synthesis and that the apparent lack of proliferation was likely not entirely due to the inability to increase cyclin A and CDK2.

What about the role of CKIs in membranous nephropathy? In contrast to a decrease in p27 levels in immune-mediated injury to mesangial cells in Thy1 [21], immune-mediated injury to GECs in membranous nephropathy was associated with a marked increase in immunostaining for the CKIs p21 and p27 [45]. Cell-type–specific markers showed that increased p21 and p27 staining localized to GECs. To show that these CKIs where responsible for limiting DNA synthesis in GECs, protein was extracted from isolated glomeruli from PHN rats, and co-immunoprecipitation studies were performed. The results showed that there was an increase in the binding of both p21 and p27 to cyclin A-CDK2 complexes in PHN rats compared with controls. Moreover, p21 and p27 inhibited the activity of CDK2 and thereby limited DNA synthesis [45].

The cytokine basic fibroblast growth factor (bFGF) is a mitogen for GECs *in vitro* [46], and giving bFGF to passive Heyman's nephritis (PHN) rats increases DNA synthesis in GECs [47]. To determine if the increase in GEC DNA synthesis was due to an increase in positive cell cycle proteins or a decrease in negative cell cycle proteins, bFGF was given to PHN rats [45]. The expression for CDK2 was not altered by bFGF. However, bFGF caused a differential down-regulation in CKI expression in PHN. The expression for the CKI p15 and p27 was unchanged. In contrast, bFGF caused a marked and selective decrease in p21 levels that coincided with increased DNA synthesis. These results showed that the increase in the CKIs p21 and p27 may be responsible for limiting DNA synthesis in the GECs following immune-mediated injury and that lowering the levels of p21 was required for DNA synthesis.

To determine the functional role for p21 in GEC proliferation, experimental glomerulonephritis was induced in p21 knockout mice and control p21 wild-type mice (see **Note Added in Proof**, number 1). A striking finding was that GEC DNA synthesis was markedly increased in nephritic p21−/− mice compared with controls. Moreover, GEC proliferation was also increased, and this was associated with increased matrix production and a marked decline in renal function compared with controls.

Figure 4 summarizes our current thinking regarding the role of CKI in GEC biology. GEC exit from the cell cycle during glomerulogenesis coincides with the *de novo* expression of the CKI p27 and p57. It is very tempting to speculate that these specific cell cycle proteins are required for GEC differentiation. In the mature quiescent GEC, the levels of CKI determine the proliferative capacity of GECs rather than the inability of GECs to increase and activate cell cycle proteins required for DNA synthesis. Thus, if the levels of p21 and p27 increase in diseases such as membranous nephropathy, GECs do not proliferate. In contrast, if p21 or p27 levels decrease following injury, GECs re-engage the cell cycle and undergo a marked increase in DNA synthesis and proliferation.

GLOMERULAR HYPERTROPHY

In contrast to the marked mesangial cell proliferation that follows many forms of immune- and nonimmune-mediated glomerular injury, the predominant growth response by the mesangial cell in diabetic nephropathy is hypertrophy [48–50]. There is also a decrease in GEC number in human diabetic nephropathy [51].

When a cell engages the cell cycle at the early G_1

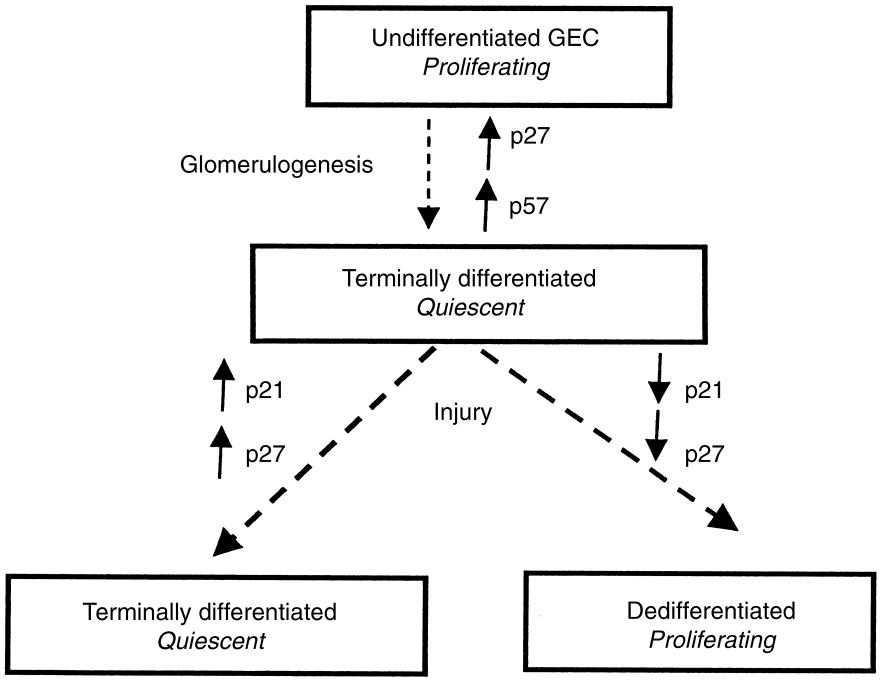

Fig. 4. Proposed schema for visceral glomerular epithelial cell (GEC) proliferation and differentiation. During glomerulogenesis, proliferating GECs exit from the cell cycle coincides with the *de novo* expression of the cyclin kinase inhibitors (CKI) p27 and p57 and the development of a terminally differentiated phenotype. The levels of CKI determine the proliferative response of mature GECs to injury. An increase in p21 and p27 maintains a terminally differentiated phenotype where GEC proliferation is limited. In contrast, lowering p21 and p27 levels is associated with a dedifferentiated phenotype, which proliferates.

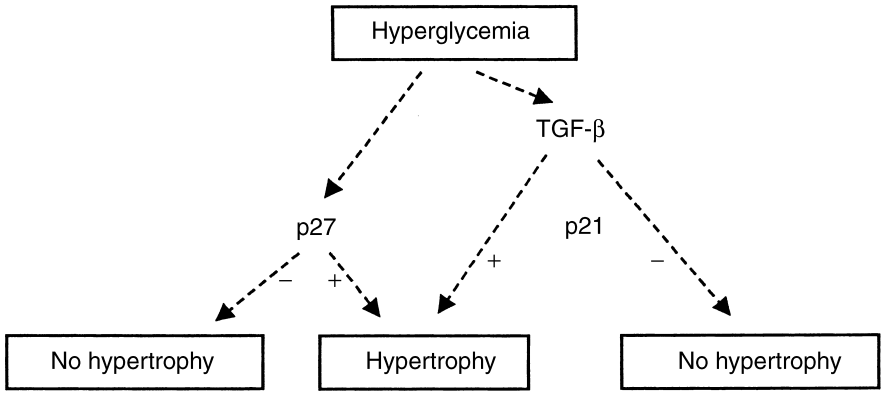

Fig. 5. Diabetic glomerular hypertrophy. The proposed schema shows that diabetic glomerular hypertrophy is due to increased levels of the cyclin kinase inhibitors p21 and p27. Hyperglycemia increases p21 and p27 levels, which block DNA synthesis in the setting where protein is increased, thus leading to hypertrophy (defined as an increase in protein to DNA ratio). Reducing levels of these cyclin kinase inhibitors attenuates diabetic glomerular hypertrophy, despite an increase in transforming growth factor-β1.

phase, there is an increase in protein content. Because of the absence of DNA synthesis during the G_1 phase of the cell cycle, the ratio of protein to DNA is physiologically increased (Fig. 1). Hypertrophy is defined as a pathological increase in protein to DNA ratio [3, 52]. Thus, we and others have developed the hypothesis that hypertrophy may be due to an increase in CKIs.

Why does mesangial cell hypertrophy predominate rather than proliferation in diabetic nephropathy? To determine if this was due to a lack of cyclin and CDK expression, studies were undertaken in cell culture and experimental diabetic nephropathy. Glucose-induced mesangial cell hypertrophy *in vitro* was not associated with an increase in levels for the late G_1 (cyclin E) or S phase (cyclin A) cell cycle proteins nor in their catalytic partner CDK2 [53]. Similarly, an increase in cyclin E and A and CDK2 could not be detected in the rat [54] and mouse [53] streptozotocin models of diabetic nephropathy.

In contrast to the expression of cyclins and CDKs, glucose-induced mesangial cell hypertrophy *in vitro* is associated with a marked increase in levels for the CKIs p21 [53] and p27 [55]. Furthermore, Wolf et al showed that lowering p27 levels with antisense oligonucleotides reduces glucose-induced hypertrophy in mesangial cells *in vitro* [55]. Similar to cell culture studies, the expression for both p21 [53] and p27 [55] is increased during glomerular hypertrophy in the streptozotocin-induced model of diabetic nephropathy, and p27 is also increased in the db/db model of diabetic nephropathy [56].

To determine if p21 is required for the development of diabetic glomerular hypertrophy, streptozotocin was

given to p21 knockout (−/−) and wild-type (+/+) mice to induce diabetes (see **Note Added in Proof**, number 2). Both groups of animals developed the same degree of hyperglycemia, and transforming growth factor-β1 (TGF-β1) mRNA levels were increased in both diabetic mouse strains. As expected, glomerular size, a measure of glomerular hypertrophy, but not cell number increased in p21+/+ mice. In contrast, despite the presence of hyperglycemia and increased TGF-β1 expression, glomerular hypertrophy was not detected in diabetic p21−/− mice. Furthermore, the loss of p21 did not convert the diabetic glomerular lesion to a proliferative state. The apparent role of cell cycle proteins in diabetic glomerular hypertrophy is summarized in Figure 5.

In summary, the role of the cell cycle regulatory proteins in glomerular disease is becoming increasingly clear. The original paradigm that the cell cycle was important only in proliferation is no longer true in glomerular and nonrenal disease. Cell cycle proteins are also closely linked with other processes that characterize glomerular disease, including hypertrophy and differentiation. Furthermore, studies have shown that certain cell cycle proteins are expressed by different cells, that the regulation may be cell-type specific, and that the results are not predictable. Of particular excitement is the likelihood that newer agents can increase or decrease the levels and activity of specific cell cycle proteins, which make them attractive potential therapeutic agents in glomerular disease.

NOTES ADDED IN PROOF

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